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Immunoblotting

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An abbreviated version of this protocol was published in Science Advances in Nov 2020

Caspase-8–dependent gasdermin D cleavage promotes antimicrobial defense but confers susceptibility to TNF-induced lethality

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Detailed protocol

Dear Marco Blasioli,

Thank you for your interest in our study. It would be a pleasure to contribute to your immunoblotting protocol with some tips. Our study mainly focused on primary bone marrow-derived macrophages, and we have not tested our immunoblotting protocol for murine RAW264.7 macrophages (each cell line gives a distinct background, and harbor a different GSDMD expression level. Thus, the protocol might need some modification). GSDMD-NT (p30) is generally generated upon processing by active caspases-1/-11/-8 (caspases-4/-5 in human). Please be sure that it is your case during your experiments (active caspases), and that you observe GSDMD full-lenght processing. Also, please be aware that GSDMD can be inactivated by caspases-3/-7, where other fragments are detected in the immunoblotting (GSDMD p43/p20). We have provided a general immunoblotting protocol in our manuscript. Here, we will describe the used protocol in further details. We hope that it will help you in your future experiments.

Cell extract preparation:

1. Prepare boiling lysis buffer (66 mM tris-Cl (pH 7.4), 2% SDS, 10 mM dithiothreitol, and NuPage LDS sample buffer; Thermo Fisher Scientific). DTT i highly sensitive to temperature, we store DTT at -20°C and avoid freeze-thaw cycles.
2. When the experiment is finished, transfer the supernatant to an 96-well plate. Remove supernatant slowly, and avoid touching the plate bottom, where cells are located.
3. Add 30 µl of boiling lysis per each 96-well plate. We plate 50.000 macrophages per well (96-well plate) one day before of the experiment. Please modify the boiling lysis volume according to your cell density.
4. Transfer the cell lysate to an Eppendorf tube and store them at -80°C.

Protein precipitation from supernatants:

1. Collect the supernatant into an Eppendorf tube (volume 1).
2. Add methanol (same volume as volume 1) and chloroform (0.3 volume from volume 1)
3. Mix samples using a vortex for 10-15 sec and centrifuge them for 10 min, 14.000 rpm, 4 °C.
4. Three phases (aqueous, interphase, organic) will be observed. Discard the upper part (aqueous) without touching the interphase (proteins).
5. Add methanol (1.3 volume from volume 1).
6. Mix samples using a vortex for 10-15 sec and centrifuge them for 10 min, 14.000 rpm, 4 °C.
7. Carefully aspirate the supernatant without touching the precipitated pellet.
8. Dry the pellets for 20-30 min at room temperature.
9. Mix the cell lysates (point 4 from cell extract preparation section) with the dried pellets for immunoblotting of combined cell extract and supernatant.

Immunoblotting

1. We perform a standard gel electrophoresis following by a transfer using Trans-Blot turbo transfer system kit (Bio-Rad) using a nitrocellulose membrane, 0.45 µm .
2. Block membrane for 1 h in an orbital shaker with blocking buffer (5 %v/v skim milk powder (local grocery store) dissolved in washing buffer 0.1 % Tween20 in 1x TBS (10 mM Tris, 150 mM NaCl, pH 7.4)).
3. Dilute the primary antibodies in blocking buffer. We normally use a 1:1000 dilution for our primary antibodies. This might strongly vary depending on the amount of cells loaded into the electrophoresis gels, and the expression level of the protein to be detected.
4. Incubate the primary antibody solution in an orbital shaker overnight at 4 °C.
5. Wash membrane with washing buffer for 2 hours on an orbital shaker at room temperature. Exchange the washing buffer four to six times.
6. Incubate the secondary antibody (we use a 1:3000 dilution (SouthernBiotech), however, dilution to be used might strongly vary depending on the provider) for 2 hours on an orbital shaker at room temperature
7. Wash membrane with washing buffer for 2 hours on an orbital shaker at room temperature. Exchange the washing buffer four to six times.
8. On a clean piece of parafilm, add 300 µl - 400 µl of each substrate for chemiluminescent substrate and mix. The amount of chemiluminescent might vary depending on the membrane size.
9. Dry the membrane with a paper tissue and place the membrane facing side with the transferred proteins on top of the substrate solution and incubate for 1 min at room temperature.
10. Remove the excess of substrate solution on the membrane with a paper tissue.
11. Place the membrane into the X-ray cassette.
12. In a dark room, place an X-ray film on top of on the membrane and close the cassette. Perform multiple exposures from 1 seconds to 30 minutes.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Demarco, B. , Chen, K. and Broz, P. (2021). Immunoblotting. Bio-protocol Preprint. bio-protocol.org/prep1070.
2. Demarco, B., Grayczyk, J. P., Bjanes, E., Roy, D. L., Tonnus, W., Assenmacher, C., Radaelli, E., Fettrelet, T., Mack, V., Linkermann, A., Roger, T., Brodsky, I. E., Chen, K. W. and Broz, P.(2020). Caspase-8–dependent gasdermin D cleavage promotes antimicrobial defense but confers susceptibility to TNF-induced lethality. Science Advances 6(47). DOI: 10.1126/sciadv.abc3465

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